

COMPOSITIONS AND METHODS  
FOR ACTIVATING GENES OF INTEREST

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Application No. 09/446,402, filed December 20, 1999, which was a National Phase Entry of PCT/US98/13093, filed June 24, 1998, which National Phase Entry claimed priority from U.S. Provisional Application No. 60/050,772, filed June 25, 1997. Each of these applications is herewith incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

The present invention relates to methods and compositions for activating genes of interest particularly in the presence of a target gene.

BACKGROUND OF THE INVENTION

The nature of and basic approaches to cancer treatment are constantly changing. At present, adjuvant chemotherapy routinely follows local treatment of cancers. Clinical protocols are now exploring genetic therapies, manipulations of the immune system, stimulation of normal hematopoietic elements, induction of differentiation in tumor tissues, and inhibition of angiogenesis. Research in these new areas has led to applications for nonmalignant disease.

At the same time, the new clinical protocols have a narrow therapeutic index as well as a great potential for causing harmful side effects. A thorough understanding of

the pharmacology, drug interactions, and clinical pharmacokinetics is essential for safe and effective use in human beings.

The therapy of viral infection is in its infancy. Bacterial infection is typically treated with agents, such as antibiotics, which take advantage of the differences in metabolism between the infecting organism and its host. However, viruses largely employed the host's own enzymes to effect the replication, and thus leave few opportunities for pharmacological intervention. By employing strong regulatory elements, the virus obtains transcription and translation of its own genes at the expense of host genes.

In mammals, viral infection is combated naturally by cytotoxic T-lymphocytes, which recognize viral proteins when expressed on the surface of host cells, and lyse the infected cells. Destruction of the infected cell prevents the further replication of the virus. Other defenses include the expression of interferon, which inhibits protein synthesis and viral budding, and expression of antibodies, which remove free viral particles from body fluids. However, induction of these natural mechanisms require exposure of the viral proteins to the immune system. Many viruses exhibit a dormant or latent phase, during which little or no protein synthesis is conducted. The viral infection is essentially invisible to the immune system during such phases.

Retroviruses carry the infectious form of their genome in the form of a strand of RNA. Upon infection, the RNA genome is reverse-transcribed into DNA, and is typically then integrated into the host's chromosomal DNA at a random site. On occasion integration occurs at a site which truncates a gene encoding an essential cellular receptor or growth factor, or which places such a gene under control of the strong viral cis-acting regulatory element, which may result in transformation of the cell into a malignant state.

Viruses may also be oncogenic due to the action of their trans-acting regulatory factors on host cell regulatory sequences. In fact, oncogenesis was the characteristic which led to the discovery of the first known retroviruses to infect humans. HTLV-I and HTLV-II (human T-lymphotrophic viruses I and II) were identified in the blood cells of patients suffering from adult T-cell leukemia (ATL), and are believed to induce neoplastic transformation by the action of their transactivating factors on lymphocyte

promoter regions. Two additional retroviruses have been found to infect humans. These viruses, HIV-I and HIV-II, are the etiological agents AIDS.

Current therapy for HIV infection includes new drugs called protease inhibitors. These drugs can dramatically reduce HIV levels in the blood when taken with other  
5 antiviral compounds such as AZT. At the same time, natural weapons in the immune system's defenses polypeptide molecules called chemokines, have been unveiled as potent foes of HIV.

Antisense oligodeoxynucleotides have been proposed as a major class of new pharmaceuticals. In general, antisense refers to the use of small, synthetic  
10 oligonucleotides resembling single-stranded DNA, to inhibit gene expression. Gene expression is inhibited through hybridization to coding (sense) sequences in a specific messenger RNA (mRNA) target by Watson-Crick base pairing in which adenosine and thymidine or guanosine and cytidine interact through hydrogen bonding.

Following the simple base-pairing rules which govern the interaction between the  
15 antisense oligodeoxynucleotides and the cellular RNA, allow the design of molecules to target any gene of a known sequence. A major advantage of this strategy is the potential specificity of action. In principal, an antisense molecule can be designed to target any single gene within the entire human genome, potentially creating specific therapeutics for any disease in which the causative gene is known. As a result, there have been numerous  
20 applications of antisense oligodeoxynucleotide (ODN) activity for potential antiviral and anticancer applications.

Antisense ODNs offer the potential to block the expression of specific genes within cells. Despite numerous reports of apparent antisense inhibition of gene expression in cultured cells, only in a few cases has specific inhibition been rigorously  
25 demonstrated. In many studies, specificity has been inferred from the biological effects of antisense as compared to control ODNs, without measuring levels of target RNA or proteins to evaluate specificity. Unintended side-effects of antisense technology could potentially occur through a number of mechanisms.

The potential of oligonucleotides as modulators of gene expression is currently  
30 under intense investigation. Most of the efforts are focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The oligonucleotides are directed

either against RNA (antisense oligonucleotides) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect, the oligonucleotides must promote a decay of the preexisting, undesirable protein by effectively preventing its formation de novo.

5           There is therefore a need for the development of new antisense methods that are more potent, reliable and specific than those used in previous studies.

### SUMMARY OF THE INVENTION

10           Compositions and methods for activating the expression of a gene of interest is provided. The compositions are antisense masked expression cassettes which comprise a double stranded nucleotide sequence. A first strand comprises an armed expression cassette, i.e., an RNA molecule which codes for a protein of interest linked downstream of a flanking sequence and a translation initiation site operably inserted upstream of the  
15   RNA sequence. The flanking sequence encodes a target molecule. That is, the flanking sequence encodes a target gene or codes for RNA of interest. The flanking sequence corresponds to the "sense" strand of the target. A second nucleotide strand is also provided, capable of hybridizing to the flanking sequence of the first nucleotide sequence; i.e., the antisense strand. The antisense strand masks the translation initiation  
20   site when bound. The flanking sequence can be designed so that the antisense sequences do not share 100% homology with the flanking sequence. Thus, in the presence of a target nucleotide molecule, the antisense strand will favor complementary binding with the target. In this manner, the antisense strand will disassociate from the armed strand and pair with the target. Disassociation of the antisense strand unmasks the ribosome  
25   binding site allowing the armed cassette to be translated in the presence of the target.

          The compositions find use in regulation of gene expression, treatment of disease, and for preventing the proliferation of neoplastic cells. Additionally, the compositions have a broad range of use in both plant and animal applications.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a diagrammatic sketch of the masked targeted expression cassette as an antiviral drug.

5        Figure 2 provides a diagrammatic sketch of the masked targeted expression cassette in which the target sequence of the sense strand is completely complementary to the antisense strand.

Figure 3 provides a diagrammatic sketch of the masked expression cassette with concatenated geometry for increasing target specificity.

10       Figure 4 provides a diagrammatic sketch of the masked targeted expression cassette with concatenated geometry which requires a quantity threshold of target molecules for initiation of translation of the desired gene.

Figure 5 provides a diagrammatic sketch of a circular masked targeted expression cassette for increased compactness and decreased viscosity.

15       Figure 6 provides a diagrammatic sketch of a stem-loop masked targeted expression system for increased compactness.

Figure 7 provides an example of a construct for production of the sense strand of the targeted cassette. The Kozak sequence is also shown (SEQ ID NO:17).

20       Figure 8 provides a diagrammatic sketch of an in vitro experiment utilizing the masked targeted expression cassette.

## DETAILED DESCRIPTION OF THE INVENTION

25       The present invention now will be described more fully hereinafter with reference to preferred embodiments. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will convey the scope of the invention to those skilled in the art.

30       Compositions and methods for controlling the expression of a gene of interest is provided. Expression is regulated by the use of antisense oligonucleotides to a target

molecule. In this manner, the gene of interest is expressed only in the presence of RNA or DNA corresponding to the target molecule.

The method involves the use of an antisense masked expression cassette. By antisense masked expression cassette is intended a double stranded nucleic acid molecule.

5 The first strand comprises an RNA molecule for the protein of interest linked downstream of a flanking sequence. The flanking sequence comprises a nucleotide sequence, the sense sequence, for a portion of the target gene. The first strand also comprises a translation initiation site downstream of the flanking sequence. It is recognized that the site of insertion for the ribosome binding site may vary. Optionally, a  
10 seven methyl guanine cap can be included to stabilize the molecule. See Figure 1.

The second strand of the masked expression cassette comprises an antisense oligonucleotide corresponding to the target gene or sequence. That is, the antisense sequence is at least partially complementary to the target sequence comprised by the flanking sequence. The antisense oligonucleotide may be either an RNA molecule, a  
15 DNA molecule or mixtures thereof. The duplex formed by binding of the second antisense strand to the corresponding flanking sequence of the first strand excludes ribosomal scanning of the downstream sequences; including the translation initiation site, the sequence of interest, or both. Thus, translation and expression of the protein of interest is masked by the binding of the antisense strand to the flanking sequence.  
20 Displacement of the antisense strand from the flanking sequence unmasks expression and translation of the protein of interest.

The protein of interest will vary depending upon the use of the composition. For example, where the masked cassette is intended as a mechanism to inhibit the growth of neoplastic cells, the protein of interest is selected from toxin proteins, cytokines, cell  
25 regulators, or the like. Additionally, the RNA molecule may be non-coding RNA, such as RNA with RNase activity.

A number of toxin proteins are known and can be used in the invention. These include ribosome inactivators, *Pseudomonas* exotoxin A (Chaudhary et al. (1990) *J. Biol Chem* 265:16303-16310); cell metabolism disruptors, such as ribonucleases (See, for  
30 example, Mariani et al. (1990) *Nature* 347:737-741); Barnase toxin, a chimeric toxin derived from *Pseudomonas* exotoxin A and a ribonuclease (Prior et al. (1990), *Cell*

64:1017-1023); Pertussis (Accession M14378 M16494, Micosia et al. (1986) Proc. Natl. Acad. Sci. USA 83:4631-4635); cholera (Dams et al. (1991) Biochim. Biophys. Acta 1090:139-141); Diphtheria, ricin (Gelfand et al. EP 0335476-A2); etc. Additionally, thymidine kinase from the herpes sequence may be used as a toxin or effector molecule.

- 5 Transcription in a cell makes it susceptible to gancyclovir. Thus, the cells of interest could be labeled and then destroyed in a two step system.

The masked cassette comprising the above described toxin sequences can be used to target and destroy any cell, organ, organism or species; so long as a target sequence can be identified that is specific to that cell, organ, organism or species. For example, the  
10 cassette can be used to selectively target and eliminate vertebrate environmental pests. Such pests include agricultural pests including foxes and rabbits in Australia.

The cassette can be used to selectively destroy cells infected with viruses. In this aspect, the target sequence comprises a virus-specific sequence, while the protein of interest is a toxin as described above.

- 15 The masked cassette can be used to treat a variety of diseases. Such diseases include, but are not limited to, diseases involving an overactive organ, such as a hyperactive thyroid. In this aspect, the masked cassette comprises a thyroid-specific target sequence and a protein toxin as described above.

The cassette can be used to treat diseases involving a defective gene. In this  
20 aspect, the target sequence comprises the sequence of the defective mRNA, while the sequence of interest comprises the sequence of the normal protein. The intended affect can be twofold. The binding of the antisense strand to the defective mRNA can shut down the production of the defective protein, while expression of the sequence of interest results in production of the normal protein.

- 25 The cassette can be used to produce a protein of interest in an organ which lacks the protein. In this aspect, the target sequence comprises an organ-specific sequence, while the sequence of interest comprises the sequence of the protein lacking in that organ.

The invention is also useful in an assay system to determine the presence of a target molecule. In this instance the protein of interest will be a reporter protein that is  
30 easily detected, for example, by either a simple cytological stain or an enzyme assay.

Such reporter sequences include but are not limited to beta galactosidase, chloramphenicol acetyltransferase (CAT), glucuronidase (GUS), and the like.

A translation initiation site is also included in the cassette. Such sequences are known in the art and include the Kozak sequence. See, for example, Kozak, Marilyn  
5 (1988) *Mol. and Cell Biol.*, 8:2737-2744; Kozak, Marilyn (1991) *J. Biol. Chem.*, 266:19867-19870; Kozak, Marilyn (1990) *Proc Natl. Acad. Sci. USA*, 87:8301-8305; Kozak, Marilyn (1989) *J. Cell Biol.*, 108:229-241; and the references cited therein. Such references are herein incorporated by reference.

The translation initiation site can be inserted upstream of the sequence  
10 corresponding to the gene of interest. Kozak sequences can be designed that can initiate translation in all three reading frames. See, for example, Murphy and Efstratiadis (1987) *Proc. Natl. Acad. Sci. USA*, 84:8277-8281. Generally, the Kozak sequence will comprise the consensus sequence recognized for initiation in higher eukaryotes. Such consensus sequence is GCCGCC<sup>A</sup><sub>G</sub>CCAUGG (SEQ ID NO:18). This consensus sequence  
15 is repeated several times within the Kozak sequence to provide for the initiation of translation in all three reading frames.

The length of the Kozak sequence may vary. Generally, increasing the length of the leader sequence enhances translation.

It is recognized that a prokaryotic translation initiation site may also be used when  
20 appropriate; for example, when targeting a prokaryote. Such sequences include the Shine-Dalgarno sequence (UAAGGAGG (SEQ ID NO:19)), typically 5-10 bases upstream of the initiator AUG.

The flanking sequence comprises a sequence which corresponds to the target gene or sequence. That is, the flanking sequence comprises all or a part of the sense strand of  
25 the target molecule and can be RNA or DNA. By sense sequence is intended a sequence capable of hybridizing to the antisense portion capable of hybridizing to messenger RNA expressed by the target when the target is a gene, or to a target RNA or DNA molecule.

The flanking sequence may vary in length. It is recognized that the length may vary depending on the length and abundance of the target gene, and the specificity and  
30 affinity of the antisense portion for the target. While the length of the flanking sequence



may vary, generally a length of about 10 to about 200 nucleotides, preferably about 20 to about 150 nucleotides, more preferably about 40 to about 100 nucleotides can be used.

The flanking sequence can be a naturally occurring or synthetic sequence. Where the sequence is synthetic, mismatch nucleotides can be incorporated into the structure to facilitate thermodynamic displacement of the antisense molecule by the target molecule. It is recognized that if the translation initiation site is inserted within the flanking sequence, this sequence insertion will provide non-hybridizing sequences and add to the decrease in homology between the flanking sequence and the antisense oligonucleotide. While it is recognized that a homology of up to 100% can be compatible with the intended displacement of the antisense strand from the flanking sequence, generally a homology of less than 90% is intended, preferably about 75% homology, more preferably about 65% homology.

A 7-methyl guanine (7MeG) cap is known to increase the efficiency of translation. Thus, such a 7-methyl guanine cap can be included on the 5' end of the flanking sequence. See, for example, Shatkin (1976) *Cell*, 9:645-653; Malone et al. (1989) *Proc. Natl. Acad. Sci. USA*, 86:6077-6081; Fuerst and Moss (1989) *J. Mol. Biol.*, 206:333-348 and Kozak (1991) *Gene Expression*, 1:117-125.

The antisense sequence of the expression cassette of the invention is constructed to hybridize with a nucleotide sequence of interest. Such nucleotide sequences of interest include messenger RNAs from target genes, viral RNAs or DNAs, and the like. The antisense strand is constructed to be homologous to the target. Generally, such homology will be greater than the homology exhibited by the antisense strand to the flanking sequence. Thus, in the presence of the target molecule, the antisense strand is displaced from the flanking sequence of the cassette and hybridizes with the target molecule. To enhance displacement, the cassette can be constructed such that the antisense sequence is longer than the flanking sequence, allowing for a 3' or 5' non-paired overhang or "sticky end" to bind the target molecule. This sticky end will enhance displacement of the antisense oligonucleotide.

As discussed, the target molecule may vary. For treatment of malignant or neoplastic cell growth, the target molecule will correspond to a nucleotide which is only expressed or present in the neoplastic cell. In this case, the sequence of interest of the

expression cassette will encode a toxin protein which is expressed in the presence of the target to kill the cell. The expression cassette could also encode a cytokine or interferon to fight neoplastic growth. In some instances, a combination of expression cassettes encoding different proteins may be provided. The target molecule can be a gene.

- 5 Numerous target genes are known in the art. Such genes include c-myc, n-myc, c-myb c-abl, c-kit, c-mos, bcr-abl, bcl-2, retinoblastoma-1, p-53, GM-CSF, G-CSF, Ick, IGF-1, egr-1 (Krieg, *ImmunoMethods* 1, 191 (1992)); c-fes (Ferrari et al., *Cell Growth Differ.* 1, 543 (1990)); c-fms (Wu et al., *Oncogene* 5, 873 (1990)); c-fos (Block et al., in (77). pp. 63-70); N-ras (Skorski et al., *J. Exp. Med.* 175, 743 (1992)); Ha-ras (Saison-Behmoaras et al., *EMBO J. MD.*, 1111 (1991)); B-myb (Arsura et al., *Blood* 79, 2708 (1992)); CSF-1 (Birchenall-Roberts et al., *J. Immunol.* 145, 3290 (1990)); Myeloblastin (Bories et al., *Cell* 59, 959 (1988)); Erythropoietin (Hermine et al., *Blood* 78, 2253 (1991)); MZF-1 (Bavisotto et al., *J. Exp. Med.* 174, 1097 (1991)); mdrl (Rivoltini et al., *Int. J. Cancer* 46, 727 (1990)); IGF-1 receptor (Porcu et al., *Mol. Cell. Biol.* 12, 5069 (1992)); Growth hormone (Weingent et al., *Endocrinology* 128, 2053 (1991)); EGR-1 (Neyses et al., *Biochem. Biophys. Res. Commun.* 181, 22 (1991)); G proteins (Supra (1992)); MHC-1# (Cambe et al., *Anti-Cancer Drug Des.* 7, 341 (1992)); Angiotensinogen (Cook et al., *Antisense Res. Dev.* 2, 199 (1992)); Myogenin (Brunetti et al., *J. Biol. Chem.* 265, 13435 (1990)); LH receptor\*\* (West et al., *Mol. Cell. Endocrinol.* 79, R9 (1991)); Cellular retinol-binding protein I, (Cope et al., in (77), pp. 125-142); TNF- $\alpha$  (A. Witsell and L. Schook, *Proc. Natl. Acad. Sci. U.S.A.* 89, 4754 (1992)).

- Target molecules include but are not limited to the CD4 gene, see, Accession No. X87579; CFTR gene (Varon et al. (1995) *Hum. Mol. Genet* 4:1463-1464); human C3d/Epstein-Barr virus receptor (Fujisaku et al. (1989) *J. Biol. Chem.* 264:2118-2125); Human MHC class I CD8 alpha-chain gene (Accession M27161, Nakayama et al. (1989) *Immunogenetics* 30:393-397); human elastase 2 mRNA (Accession M16631), Fletcher et al. (1987) *Biochemistry* 26:7256-7261); Human elastin mRNA (Accession M36860, Fazio et al. (1988) *J. Invest. Dermatol.* 91:458-464); human intercellular adhesion molecule 1 gene (Accession U86814); human interleukin 1-beta converting enzyme isoform beta mRNA (Accession U13697 Alnemri et al. (1998) *J. Biol. Chem.* 270:4312-4317); human immunoglobulin C mu-C delta locus (Accession X57331, Word et al.

(1989) *Int. Immunol* 1:296-309); human interleukin 2 gene (Accession J00264, Maeda et al. (1983) *Biochem. Biophys. Res. Commun.* 115:1040-1047; Fujita et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:7437-7441); human MHC Class I antigen HLA-B (Accession U88407); human MHC class II HLA-DPA1 antigen (Accession U87556); etc. herein  
5 incorporated by reference.

Likewise, the target molecule may be a RNA or DNA from a virus. In this manner, viral replication and growth can be inhibited. Such viral genes include but are not limited to sequences from Coxsackievirus (Marquardt and Ohlinger (1995) *J. Virol. Methods* 53:189-199); Dengue virus, see Accession No. U88535; encephalitis virus, see,  
10 Accession No. AB001026; Ebola virus (Sanchez et al. (1989) *Virology* 170:81-91, Accession No. L11365); Epstein-Barr virus (Baer et al. (1984) *Nature* 310:207-211); Echovirus 32 (Huttunen et al. (1996) *J. Gen. Virol.* 77:715-725); Enterovirus (VP4-VP2 capsid 3D RNA polymerase genes Pulli et al. (1995) *Virology* 212:30-38); influenza A virus (Guan et al. (1996) *J. Virol.* 70:8041-8046); hepatitis B virus (Fukuda et al. (1995)  
15 *J. Infect. Dis.* 172:1191-1197); hepatitis C virus (Hitomi et al. (1995) *Viral Immunol.* 8:109-119); hepatitis D virus (Khudyakov et al. (1993) *Virus Res.* 27:13-24); hepatitis E virus (Tam et al. (1990) *Science* 247:1335-1449, Accession No. M32400); hepatitis G virus (Accession No. U86023); HIV (Accession U04908, Gao et al. (1996) *J. Virol.* 70:1651-1667); human papillomavirus (Accession U37537, Wu et al. (1993) *Lancet*  
20 341:522-524); influenza A virus (Accession U86987); human rhinovirus (Accession D00239, Hughes et al. (1988) *J. Gen. Virol.* 69:49-58); Sendai virus (Accession D00053 N00053, Morgan and Rakestraw (1986) *Virology* 154:31-40); gastroenteritis virus TFI virion protein gene (Accession Z35758; Chen et al. (1995) *Virus Res.* 38:83-89); herpes simplex type 2 virus (Accession Z86099, McGeoch et al. (1987) *J. Gen Virol.* 68:19-38);  
25 Venezuelan equine encephalitis virus (Accession L01442, Kinney et al. (1986) *Virology* 152:400-413); herein incorporated by reference.

Other genes of interest include, for example, *jun*, *bFGF*, *wnt-1*, *TGF-beta*, *spi-1* for cytomegalovirus; *NDR*, *c-erbB-2* for herpes simplex virus, types 1 and 2; *bcl-2* and *bcl-abl* for human papilloma virus; *p53* and *c-myc* for hepatitis, type B; *l-myc* and *ras* for  
30 influenza virus; etc.

Methods are generally available in the art for construction of the masked expression cassettes. See, for example, Sambrook et al., Cold Spring Harbor, NY. RNA/DNA molecules as well as antisense oligonucleotides can be made in accordance with known techniques. See, e.g., U.S. Patent No. 5,149,797; 5,175,273; Uhlmann and  
5 Peyman (1990) Chem. Rev., 90:543-584 and the references cited therein. The antisense oligonucleotides, which may be deoxyribonucleotide or ribonucleotide sequences which are capable of complementary binding to the target molecule. Such antisense oligonucleotides may be oligonucleotides wherein at least one, or all, of the internucleotide bridging phosphate residues are modified phosphates, such as methyl  
10 phosphonates, methyl phosphonothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, some, for example, every other one, of the internucleotide bridging phosphate residues may be modified as described. In another example, such antisense oligonucleotides are oligonucleotides wherein at least one, or all, of the nucleotides contain a 2' loweralkyl moiety (e.g., C1-  
15 C4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). See also Furdon et al. (1989) Nucleic Acids Res., 17:9193-9204; Agrawal et al. (1990) Proc. Natl. Acad. Sci. USA, 87:1401-1405; Baker et al. (1990) Nucleic Acids Res., 18:3537-3543; Sproat et al. (1989) Nucleic Acids Res., 17:3373-3389; Walder and Walder (1988) Proc. Natl. Acad. Sci. USA,  
20 85:5011-5015.

Modification of the phosphodiester backbone has been shown to impart stability and may allow for enhanced affinity and increased cellular penetration of ODNs. Additionally, chemical strategies may be employed to replace the entire phosphodiester backbone with novel linkages. Phosphorothioate and methylphosphonate modified  
25 ODNs may be made through automated ODN synthesis.

A phosphorodithioate version of the phosphorothioate can be synthesized. In the dithioate linkage, the non-bridging oxygens can be substituted with sulfur. This linkage is highly nuclease resistant.

Sugar modifications may also be used to enhance stability and affinity of the  
30 molecules. The alpha-anomer of a 2'-deoxyribose sugar has the base inverted with

respect to the natural beta-anomer. ODNs containing alpha-anomer sugars are resistant to nuclease degradation.

If necessary, targeted cassette can be modified to increase stability in vivo. Thus, nuclease-resistant oligonucleotides can be utilized, such as PS and MP oligonucleotides.

5 See, for example, Miller (1991) *Biotechnology*, 9:358 and Stein et al. (1991) *Pharmacol. Ther.*, 52:365.

The targeted expression cassettes of the invention can be synthesized easily and in bulk. The development of phosphoramidite chemistry and its elaboration into an automated technology have greatly enhanced the ease with which oligos are synthesized  
10 and consequently their availability. See, for example, Beaucage and Caruthers (1981) *Tetrahedron Lett.*, 37:3557 and Zon and Geiser (1991) *Anti-Cancer Drug Des.*, 6:539.

The methods, oligonucleotides and formulations of the present invention have a variety of uses. They are useful in preventing the proliferation and growth of neoplastic cells. The methods, oligonucleotides and compositions of the present invention are also  
15 useful as therapeutic agents in the treatment of disease. They also find use in fermentation processes where it is desirable to have a means for regulating the expression of a gene to be expressed at a certain time or any instance where it is desirable to regulate gene expression.

The term "antisense oligonucleotides" includes the physiologically and  
20 pharmaceutically acceptable salts thereof: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. Examples of such salts are (a) salts formed with cations such as sodium, potassium, spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the  
25 like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from  
30 elemental anions such as chlorine, bromine, and iodine.

Formulations of the present invention comprise the masked cassette in a physiologically or pharmaceutically acceptable carrier, such as an aqueous carrier. Thus, formulations for use in the present invention include, but are not limited to, those suitable for parenteral administration, including subcutaneous, intradermal, intramuscular, intravenous and intra-arterial administration, as well as topical administration (i.e., administration of an aerosolized formulation of respirable particles to the lungs of a patient afflicted with cystic fibrosis). The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art. Such formulations are described in, for example, Remington's Pharmaceutical Sciences 19th ed., Osol, A. (ed.), Mack Easton PA (1980). The most suitable route of administration in any given case may depend upon the subject, the nature and severity of the condition being treated, and the particular active compound which is being used.

The present invention provides for the use of the targeted masked cassette having the characteristics set forth above for the preparation of a medicament for the various disorders. In the manufacture of a medicament according to the invention, the masked cassette is typically admixed with, inter alia, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid. One or more antisense oligonucleotides may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

Formulations of the present invention may comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of intended recipient and essentially pyrogen free. These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring

only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

In the formulation the targeted cassette may be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which may be suitable for parenteral  
5 administration. The particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the targeted cassette is contained therein. Positively charged lipids such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-amoniummethysulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. See, e.g., U.S. Patent Nos.4,880,635 to Janoff et al.;  
10 4,906,477 to Kurono et al.; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen et al.; 4,921,757 to Wheatley et al.; etc.

The dosage of the targeted cassette administered will depend upon the particular method being carried out, and when it is being administered to a subject, will depend on the disease, the condition of the subject, the particular formulation, the route of  
15 administration, etc. In general, intracellular concentrations of the cassette of from .05 to 50 $\mu$ M, or more particularly .2 to 5  $\mu$ M, are desired. For administration to a subject such as a human, a dosage of from about .01, .1, or 1 mg/Kg up to 50, 100, or 150 mg/Kg is employed.

Current technology has focused on antisense molecules only. Antisense  
20 oligonucleotides bind the offending RNA molecules in the cell. To be effective, a high dosage of antisense molecules have to be delivered to each cell. The present invention provides for an effector molecule which increases the potency of antisense technology. In this manner, the cell can be manipulated more easily and a far lower dosage, potentially even a 1 molecule to 1 cell ratio can be effective.

It is the idea of specificity that provides the underlying feature of the present  
25 invention. Standard cytotoxic chemotherapy for conditions such as neoplastic disease is fraught with systemic toxicity. The ratio of the toxic dose to the therapeutic dose is relatively low, which reflects the large number of cellular targets affected by the chemotherapeutic agent and the agents inability to distinguish between normal and  
30 diseased cells. In theory, this problem is solved by taking advantage of the specificity conferred by Watson-Crick base pair formation by identifying an appropriate target.

The following experiments are offered by way of illustration and not by way of limitation.

### Experimental

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#### Use of Targeted Cassette to Kill Neoplastic Cells

Following the protocols as essentially described above, a targeted cassette is constructed wherein the first strand has an RNA coding for toxin A. The toxin RNA is linked with upstream DNA sequences coding the sense portion of the p53 DNA  
10 sequence. The p53 protein is found in numerous cancer cells. Inserted within the p53 molecule is a Kozak sequence. An antisense structure is constructed which corresponds to the p53 sense nucleotide.

The targeted cassettes are provided to a patient in a pharmaceutically acceptable solution at a concentration of from about .01, .1, or 1 mg/Kg up to 50, 100, or 150  
15 mg/Kg.

#### Use of Targeted Cassette as Antiviral Drug

Figure 1 depicts utilization of the masked targeted expression cassette as an antiviral agent (Black RNA Drug). Features of the cassette are identified by the provided  
20 key. The sense strand has a 7meG cap at its 5' end. In the inactive form, the antisense strand is hybridized to the flanking sequences of the sense strand; such that the Kozak sequence is masked. Mismatch area between the Kozak sequence and the antisense strand is indicated by lack of hydrogen bonding. Upon presentation of active viral RNA which has perfect homology to the antisense strand, the antisense strand dissociates from  
25 the sense strand and binds the viral RNA, which renders the viral RNA inactive. Furthermore, upon dissociation of the antisense strand, the Kozak sequence is unmasked and translation of the toxin protein commences from the AUG initiation codon. Upon production of toxic quantities of mature toxin, the cell hosting the virus is destroyed.



### Targeted Cassette with Totally Complementary Antisense and Flank Sequences

Figure 2 depicts a masked targeted expression cassette in which the viral target sequence of the sense strand is completely complementary to the antisense strand. In the inactive targeted cassette, ribosomal assembly and scanning from the 5' end is prevented by the duplex between the antisense strand and the flanking sequence. In this example, displacement of the antisense strand and activation of expression of the gene of interest (lac Z) can be tested by assaying for  $\beta$ -galactosidase activity.

### Targeted Cassette with Increased Target Specificity

Figure 3 depicts a masked targeted expression cassette with concatenated geometry for increasing target specificity for initiation of translation of the gene of interest. Each antisense/sense combination (1-3) corresponds to a different target sequence. For example, 1 corresponds to a viral RNA, 2 corresponds to a cytokine RNA, and 3 corresponds to a host specific protein. RNA encoding the protein of interest is only expressed when all 3 target sequences are present in the target cell, effecting displacement of all 3 antisense sequences from the sense sequences of the cassette, thereby allowing ribosomal assembly and scanning from the 5' end to proceed to the Kozak sequence and the AUG start codon.

### Targeted Cassette with Target Quantity Threshold

Figure 4 depicts a masked targeted expression cassette with concatenated geometry for requiring a target quantity threshold for initiation of translation. All sense/antisense combinations (1-3) correspond to the same target RNA. RNA encoding the protein of interest is only expressed when the target RNA is present in sufficient quantity (in this example 3 copies) in the target cell, effecting displacement of all 3 antisense sequences from the sense sequences of the cassette, thereby allowing ribosomal assembly and scanning from the 5' end to proceed to the Kozak sequence and the AUG start codon. The concatenated geometry thus requires a threshold quantity of the target RNA for initiation of translation. Such concatenated cassette constructs are particularly useful for targeting cancer cells with abnormally high number of copies of a particular mRNA. The constructs may also be made in combination with the constructs of Figure 3.

### Circular Targeted Cassette

Figure 5 depicts a circular masked targeted expression cassette.

An antisense molecule complementary to a target molecule is bound to complementary sequences at the 3' and 5' end of the targeted expression cassette, thereby preventing ribosome assembly and scanning from the 5' end. Thus, displacement of the antisense strand in the presence of a complementary target molecule allows for translation and expression of the desired protein. The circular configuration may be more compact and less viscous, thereby having particularly desirable properties for drug delivery applications.

### Stem-loop Targeted Cassette

Figure 6 depicts a stem-loop masked targeted expression cassette. An antisense molecule complementary to a target molecule is bound to complementary sequences within the loop structure, further stabilizing the stem-loop structure. Ribosomal scanning from the 5' end is prevented from proceeding to the Kozak sequence and the initiation AUG codon by the stable secondary structure of the complex. Displacement of the antisense strand in the presence of a complementary target molecule provides a less stable stem-loop structure unable to prevent ribosomal scanning commenced from the 5' end.

### Armed Sense Strand Plasmid Construct.

Figure 7 depicts a pCI-Neo (Promega Corp.) plasmid construct for production of the sense RNA strand of a targeted expression cassette. The complete sequence of the depicted MCS-Kozak-lac Z is set forth in SEQ ID NO: 1. Alternative flanking sequences corresponding to portions of the firefly luciferase mRNA are inserted into the multiple cloning site (MCS), such that transcription from the T<sub>7</sub> promoter yields RNA comprising from the 5' end; luciferase segment-Kozak-β gal. The alternative luciferase sense segments are set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7 or 8.

In vitro determination of activity of masked targeted expression cassette.

Sense strand RNA of the masked targeted cassette is produced by in vitro transcription of the construct depicted in Figure 7, by use of the Riboprobe® Combination System (Catalogue No. P1450, Promega Corp.).

5 Antisense sequences corresponding to portions of the target molecule (firefly luciferase RNA, Catalogue No. L4561, Promega Corp.) are hybridized to complementary flanking sequences of the sense strand of the targeted cassette. SEQ ID NO: 2, 3, 4, 5, 6, 7 or 8 list alternative flanking sequences and SEQ ID NO: 9, 10, 11, 12, 13, 14 or 15 list the corresponding antisense sequences, respectively. The full length firefly luciferase  
10 RNA, according to which the flanking sense sequences and corresponding antisense sequences are made is set forth in SEQ ID NO: 16. The hybridized mixture is introduced to an in vitro translation mixture containing ribosomes and full length firefly luciferase RNA (Flex®, Rabbit Reticulocyte Lysate System, Catalogue No. L4540, Promega Corp.). Control reactions will lack the masked cassette.

15 After completion of translation, the mixture will be assayed for  $\beta$ -galactosidase ( $\beta$ -gal) and luciferase activities. Negative luciferase and positive  $\beta$ -gal activity indicates successful inhibition of the target molecule and successful expression of the gene of interest.

The diagram depicts the mechanism of the assay. In panel 1, the antisense  
20 sequence is bound to complementary flanking sequence of the targeted cassette. Ribosomal scanning commenced from the 5' end is blocked by the antisense/sense duplex, thereby preventing translation of the  $\beta$ -gal RNA. Displacement and binding of the antisense to target luciferase RNA (panel 2) has a two-fold effect (panel 3).  $\beta$ -gal can be expressed from the unmasked cassette ( $\beta$ -gal positive) and expression of the target is  
25 blocked by binding of the antisense to the target (luciferase negative).

Other modifications and embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings presented herein. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed. Although specific terms are employed, they are  
5 used in generic and descriptive sense only and not for purposes of limitation, and that modifications and embodiments are intended to be included within the scope of the appended claims.